AD_____ (12)

MECHANISM OF CHEMICAL ACTION AND TREATMENT OF CYANIDE POISONING

Final Report

Charles A. Tyson, Ph.D.

1 August 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2211
SRI Project LSU-4768

SRI International
333 Ravenswood Avenue
Menlo Park, California 94025

AD-A186 759

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY: Unclassified	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: SRI International; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 333 Ravenswood Avenue, Menlo Park, CA 94025	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-82-C-2211
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M1-62734A875; TASK NO. AJ; WORK UNIT ACCESSION NO. 117

11. TITLE (Include Security Classification)
Mechanism of Chemical Action and Treatment of Cyanide Poisoning

12. PERSONAL AUTHOR(S)
Charles A. Tyson, Ph. D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final Report	FROM 82/9/1 TO 86/7/28	1986 August 1	29

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Potassium cyanide, cytotoxicity, hepatocyte-erythrocyte coincubation system, methemoglobin antidotal mechanism,
06	15		
07	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This contract was concerned with the development, validation, and use of a coincubation system composed of hepatocyte monolayer cultures and erythrocytes in suspension in the culture medium for study of cyanide and antidotal mechanisms and for preliminary screening of newer antidotes. We developed a simple apparatus that was convenient and inexpensive to use. We found that KCN addition to the medium depressed O_2 consumption and related functional parameters (ATP, ATP/ADP, and urea synthesis) in rat hepatocytes selectively as expected and at concentrations that were relevant to those that produced analogous effects on liver tissues of rats given the toxin in vivo. Effects of short-term exposures to cyanide in the cultures were reversible by replacing the medium with one containing no cyanide or by adding known cyanide antidotes directly to the medium. Thus, addition of 4-dimethylaminophenol, cobalt(II) chloride, sodium nitrite, sodium thiosulfate, or a combination of the last two antidotes to the medium reversed KCN-induced depression of hepatocyte ATP in a concentration-dependent manner. The relative effectiveness of these

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Judy Pawlus	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

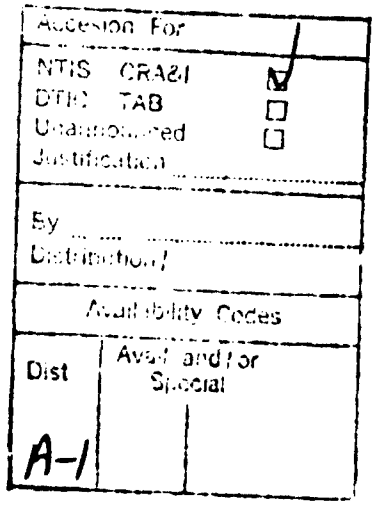
18. (cont.) thiosulfate:cyanide sulfurtransferase activity, rhodanese, sodium nitrite, 4-dimethylaminophenol, cobaltous chloride, sodium thiosulfate, cis-diaquo-bis(2-2'-bipyridyl)Co(III) triperchlorate (BK57874), 2-aminoethanethiol (BE15684), 3-(4-aminobutylamino)propyl mercaptan (BK71025), 2-(3-aminopropylamino)ethyl mercaptan (BK71365), 2-(5-aminopentylamino)ethyl mercaptan (BK73869), 4-methyl-8-diethylaminohexylquinoline (WR6026), primaquine (WR2975).

19 (cont.) antidotes in antagonizing cyanide action on hepatocytes was grossly similar to their relative efficacy in vivo in preventing cyanide-induced lethality. The coincubation system, validated in this way, was used to test mechanistic hypotheses on the modes of cyanide and antidotal action at the cellular and molecular levels, to screen for interspecies differences in response using cells derived from dog (beagle) and primate (rhesus monkey and human) tissues, and to test other antidotes and drugs supplied by the Walter Reed Army Institute of Research.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.



CONTENTS

TABLES

FIGURES

Introduction

This contract was concerned with the development, validation, and use of *in vitro* systems for the study of cyanide and antidotal mechanisms at the cellular level and the preliminary screening of antidotes for potential effectiveness. These systems are intended to provide a sensitive, economical means with which to study the effects of cyanide (KCN) and its antidotes on isolated target cells--information not obtainable from *in vivo* studies. Interspecies comparisons are possible using tissues from rodents and larger animals, including humans. Various cytotoxicity indicators were monitored for insight into the mechanism of action of cyanide and use in the antidotal studies.

The antidotes studied in the system for validation and mechanistic information are 4-dimethylaminophenol, sodium nitrite, cobalt complexes, and sodium thiosulfate. Recent reviews have summarized the state of knowledge on the action of cyanide and these antidotes (1-5).

In Vitro Systems Development

Initially, the work was focused on the development of a system comprising hepatocytes and erythrocytes suspended in a physiologic-like medium in separate but interconnected compartments to assess the practicability of the approach. A semipermeable membrane would allow solutes to migrate between the compartments by passive diffusion. Hepatocytes were chosen as a target cell for cyanide action primarily out of an interest in documenting a role for liver enzymes in cyanide detoxication. Erythrocytes were required to provide hemoglobin for those antagonists that induce methemoglobin formation for complexation with cyanide. These cell types can be readily isolated from different mammalian species including human for comparative studies. The medium contained serum albumin, which has been implicated in detoxication by sulfur transfer to cyanide.

After we had tried several types of reaction flasks, both commercial and SRI-designed and -made, semipermeable membranes with different pore sizes and densities and various shaking rates, it became clear that the original approach had a serious technical limitation. We found that the time for equilibration between compartments, as measured by tritiated water diffusion, was too long (at least 20 min). To shorten the time considerably, active pumping or cycling of the medium between compartments would have to be employed, which methodology would clearly entail a more elaborate, expensive, and cumbersome setup. This undertaking was deemed impractical in terms of cost and effort for development and validation.

We then tried a simpler system, in which hepatocytes were attached to collagen-coated, plastic tissue culture dishes, a technique commonly employed in our and other laboratories for toxicity and metabolism studies (6). Erythrocytes were added in suspension to the medium. The culture dishes were placed inside an airtight reaction flask, and incubations were conducted in a shaking water bath at 37°C under an air:CO_2 (95:5)

atmosphere for the designated reaction time (usually 1 hr maximum). The system is shown in Figure 1.

The compatibility of hepatocytes and erythrocytes in this system was demonstrated in incubations lasting up to 2 hr. Release of lactate dehydrogenase (LDH) from the cells to the medium, an indication of loss of plasma membrane integrity, was less than 5% during this period, indicating no significant deterioration in this property under the incubation conditions. The sum of the LDH in the medium of separate flasks containing either hepatocytes or erythrocytes alone was approximately equal to the LDH in the medium of the complete system. In addition, ATP levels were not significantly altered in either cell type during the incubation. On the basis of these findings, the system appeared to be suitable for use in testing the action of cyanide and its antidotes on the cells.

Cyanide-Induced Cytotoxicity

The effects of KCN on the cell components of the system were investigated with a view toward further validating the approach, acquiring data that might be useful for mechanistic interpretations (6-8), and determining the best cytotoxicity indicator for use in antidotal studies. Criteria adopted for demonstrating validity were that cyanide should be selectively cytotoxic to hepatocytes, because these possess mitochondria--in contrast to erythrocytes, which do not--and that cyanide should inhibit mitochondrial respiration at concentrations similar to those *in vivo* that produce cytochrome oxidase inhibition in liver tissue. Commonly used cytotoxicity indicators evaluated for use in the antidotal studies were hepatocyte ATP (and ATP/ADP) levels, urea synthesis rates, lactate/pyruvate (L/P) and 8-hydroxybutyrate/acetoacetate (BHB/ACET) ratios, and LDH release. Additional data acquired for determining the involvement of other factors in hepatotoxicity included measurements of O_2 consumption, reduced glutathione levels, and lipid peroxidation in the hepatocytes and release of aspartate aminotransferase (AST) (for mitochondrial and plasma membrane integrity) and acid phosphatase (AP) (for lysosomal membrane integrity) to the medium.

Alternatively, the effect of KCN on O_2 consumption was assessed in hepatocyte suspensions since the measurement cannot be made conveniently with hepatocyte cultures using polarographic techniques, the method of choice (8). Separate experiments with hepatocyte suspensions and monolayer cultures in the absence of erythrocytes in the medium showed that the addition of KCN induced the same response of ATP depression and LDH release. This finding is evidence that the results in the two systems were essentially interchangeable. A Yellow Springs oxygen monitor was used for the polarographic measurements, and the technique is simple and straightforward. Briefly, the baseline rate of O_2 consumption for the cells was established by placing the electrode holder into the water-jacketed glass reaction cylinder, thus shutting off contact with the external atmosphere. Then KCN solution was added by microsyringe through a port in the holder. The rate of O_2 consumption (indicated by the tracing) slowed appreciably, reaching a new linear rate within 15 to 45

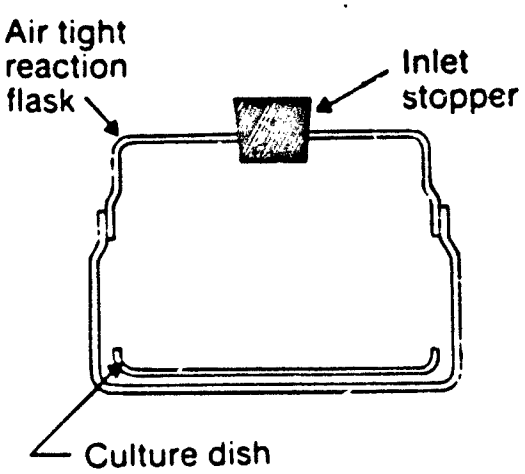


Figure 1. Reaction Flask Used for Coincubation System. In complete system, culture dish inside contains hepatocyte monolayer (1.5×10^5 cells/dish) in culture medium supplemented with hormones and 0.2% bovine serum albumin.

sec, depending on the cyanide concentration. These results were consistent with the expected role of cyanide in inhibiting mitochondrial respiration to impair the functional capabilities of the cell. After the erythrocytes were removed by centrifugation, no free hemoglobin was detected in the medium to indicate cell lysing.

To validate the system for cyanide studies further, we compared concentrations of the toxin in the medium that inhibited mitochondrial respiration (the most sensitive change detected among the indicators monitored; see below), with circulating levels at lethality. The data compared were obtained from literature reports. A plot of *in vitro* data, shown in Figure 2, indicated the EC50 value for inhibition of O_2 consumption to be 78 μM (8). This value is very similar to the K_m for half-maximal complexation of cyanide with rat brain cytochrome oxidase (50 μM) determined *in situ* (9), as would be expected for a general cytotoxin like cyanide, which freely permeates tissues and acts on the same target site in cells. Also, the circulating level of cyanide in rat plasma at lethality is estimated to range from 30 μM, the minimum value reported, to 120 μM, at which level significant numbers of the animals die (4,10,11). Liver cytochrome oxidase in rat, mouse, and rabbit have been shown to be inhibited at lethal doses of cyanide (4,12-14). From these data and estimates, we concluded that the concentration ranges of cyanide producing inhibition of mitochondrial respiration in isolated hepatocytes and in liver cells *in vivo* overlap, a good indication that mechanistic studies of this toxin in the *in vitro* system have relevance in animals *in vivo*.

The addition of KCN to hepatocyte monolayer cultures caused time- and concentration-dependent changes in several parameters (6,7). The earliest-detected changes were depressed O_2 consumption and cellular ATP (within minutes). These were followed by increased L/P (and BHB/ACET) and decreased urea accumulation in the medium (within 30 min); intracellular enzymes were released to the medium much later (1 to 4 hr). At low cyanide concentrations (0.10 and 0.20 mM), the data in general suggested a close temporal relationship between inhibition of O_2 consumption and ATP depression. At high cyanide concentrations (0.50 mM and above), ATP depression lagged behind inhibition. This presumably reflects the time required for dissipation of the adenine nucleotide by metabolic processes in the cell, since the bulk of the ATP (60%) is extramitochondrial. An observed delay in L/P and urea synthesis response probably stems in part from insensitivity in detection because of methodological limitations. In contrast to these observations, reduced GSH levels, lipid peroxidation (assessed by measuring thiobarbituric acid reactants in the hepatocytes), and leakage of intracellular enzymes AP, LDH, or AST were either unchanged up to cell death or late events.

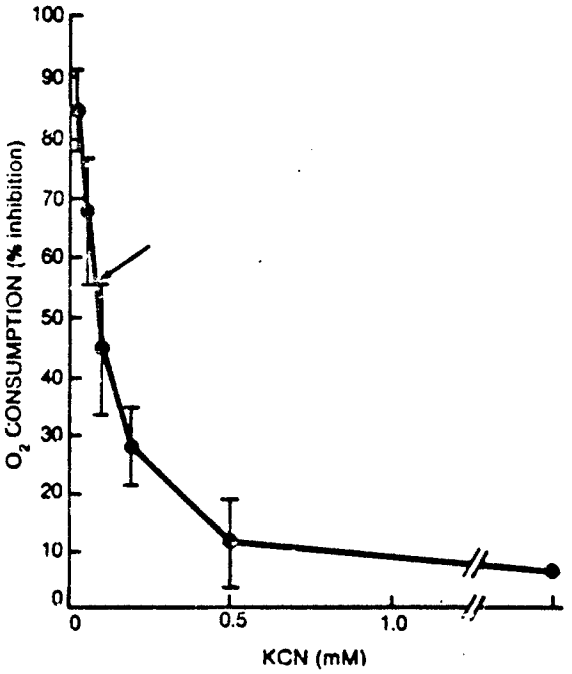


Figure 2. Percent Inhibition of O_2 Consumption in Rat Hepatocytes as a Function of KCN Concentration. Experimental conditions are the same as with monolayer culture except that the measurements are made on hepatocyte suspensions polarographically. Value for K_m is indicated by arrow and applies to mitochondrial component of total O_2 consumption.

In addition to being among the earliest, if not the earliest, changes detected, inhibition of O_2 consumption occurred at cyanide concentrations lower than those required for the other cytotoxicity indicators, and in greater magnitude. These observations are consistent with the hypothesis that this is the critical event initiating toxicity. Thus, the half-maximal concentrations for changes in ATP levels, ATP/ADP, and urea synthesis rates (a process also intimately dependent on mitochondrial respiration) were estimated to be 0.20-0.24, 0.14, and 0.11 mM, respectively, somewhat greater than the K_m for inhibition of O_2 consumption by cyanide (8). The reason for the difference in values is that the cell compensates for the lower ATP levels by synthesizing more ATP through unblocked oxidative phosphorylation pathways in the mitochondria and through glycolysis, which also contributes to ATP generation, though less efficiently. These observations are consistent with the known response of these indicators in target tissues in vivo to hypoxic conditions and to cyanide. However, since LDH release occurs at higher concentrations yet ($\geq$ 0.50 mM), it is not possible to exclude direct effects of cyanide on cell processes or enzymes in addition to energy conservation as contributing to cell death.

Some alternative mechanisms of cell death were evaluated and dismissed because of a lack of supporting data. Thus, GSH levels were not depressed and lipid peroxidation was not detected in hepatocytes treated with cyanide up to the point of cell death. The possibility that cyanide might form cyanohydrins with Schiff bases, disrupting intermediary metabolism (5,15), was considered. If this were true, one would expect a depression in total AST, which enzyme requires pyridoxal-5'-phosphate as a cofactor at cytotoxic concentrations of cyanide. In an earlier study with rat hepatocyte suspensions (16), we acquired data addressing this point. Thus, total AST (medium + cells) was depressed 20 and 30% after 2- and 5-hr incubations of the hepatocytes with 1.0 mM KCN and only 5% at most with 0.10 mM KCN in the medium; total LDH was not appreciably changed. This apparently selective action on AST is tentatively attributed to cyanohydrin formation with the Schiff base cofactor at the active site of the enzyme. However, because the change in total AST was far less than LDH release (81%) at 5 hr in those experiments, the change appears to be relatively minor and unrelated to the mechanism of cell death. It must be cautioned that this interpretation is tentative because of dilution of the aliquot and addition of cofactor itself in performing the assay (17), which may result in some reversion of the cyanide-induced inhibition. It may also be relevant that cyanide produced no appreciable change in intermediary metabolism of erythrocytes (based on unchanged L/P and ATP levels in experiments with these cells only) or cell death at the same concentrations that did so in hepatocytes.

An obviously late event on prolonged incubation of the hepatocytes with KCN is release of intracellular enzymes to the medium (Figure 3). Significant LDH release above control cell release, an indication of total loss of plasma membrane integrity and cell death, was not detected until 2 and 4 hr after addition of KCN ($\geq$ 0.50 mM). (AP release responded

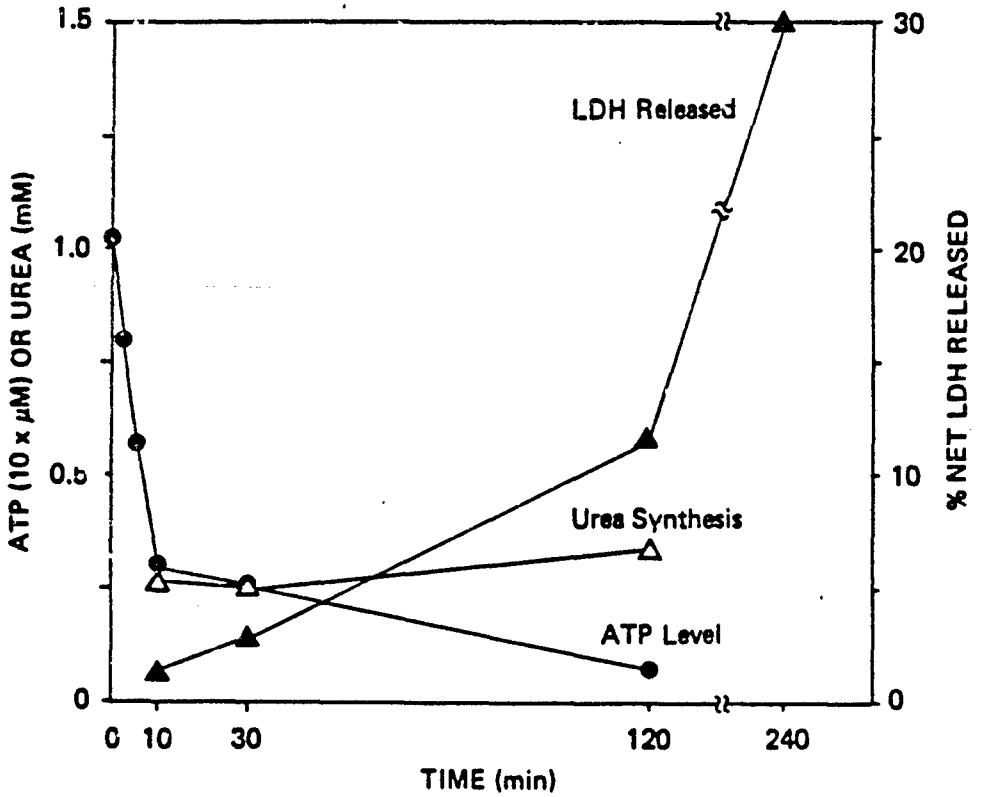


Figure 3. Time Course Changes in Cytotoxicity Indicators in Hepatocyte Monolayer Culture in Response to 1.0 mM KCN.

similarly to LDH release.) Under these conditions, ATP levels (and related measures of the energy charge status of the cells--ATP/ADP or energy charge potential) drop below the level required for maintenance of ion gradients needed for cell survival and remain there long enough for irreversible injury to the cells (18). The results offer an explanation of why release of liver enzymes to the serum is not ordinarily observed in acute cyanide poisoning, in that the time for signs of liver necrosis to become evident is much longer than that for lethality, which occurs within minutes. In contrast to AP and LDH release, ≤ 25% AST was released under the same conditions (data not shown in figure), suggesting that the mitochondrial inner membrane had remained intact.

Although brain, not liver, is the primary target organ for cyanide poisoning, the results of these studies bear on the mechanism of cyanide toxicity in vivo. Overall, the results obtained with isolated hepatocytes, together with what is known about the interaction of cyanide with cell components (15) and from in vivo studies (19-22), are totally consistent with the hypothesis that cytochrome oxidase inhibition is the primary event leading to toxicity. Way et al. (5) noted that the lethal effects of cyanide occur at circulating levels that far exceed the minimal concentration to inhibit this enzyme (e.g., 15), and Pettersen and Cohen (23) found that the degree of inhibition in target tissues at lethal and nonlethal doses is similar, raising the possibility that mechanisms other than cytochrome oxidase inhibition may be involved in lethality. However, it should be emphasized that it is not inhibition per se but the consequence of the stress induced by the inhibition on other cellular processes that underlies toxicity. Because of its high metabolic rate and meager reserves of high-energy phosphates, brain tissue is more critically dependent on O_2 supply and respiration than heart or liver (24-26). We suggest that functional deficits or their magnitude produced by the oxidative stress caused by cytochrome oxidase inhibition and the consequences for survival of the organism may be different in target tissues. However, because we do not have a comprehensive understanding of these processes, as has been pointed out (5,23), additional research to identify and define them more precisely is needed.

The choice of the best indicator from among those studied for use in experiments with antidotes involved several considerations. Short-term exposure of target tissues to cyanide is reversible (18), and the indicator chosen must likewise be "reversible" to reflect antidotal antagonism in protocols simulating prophylactic treatments. In addition, the indicator should be convenient to measure, relatively sensitive, reproducible, and capable of assessing the status of the cell instantaneously at various time points, as opposed to being a rate measurement that requires several minutes to record. O_2 consumption was eliminated from consideration because it cannot be measured conveniently in hepatocyte cultures, a requirement for maintaining separation of the two cell types in the system developed. Urea synthesis, though specific for hepatocytes, was inappropriate for antidotal studies because of the long incubation time required for its measurement. Although fairly sensitive, L/P was not specific to hepatocytes and was sluggish in response to control cell

levels when cyanide was removed from the medium. BHB/ACET in the medium is hepatocyte-specific, but changes in this parameter were less sensitive and reproducible than those in hepatocyte ATP. The latter indicator meets most of the above criteria. However, in the combined system erythrocyte ATP contributes about two-thirds of the total under the conditions used, reducing sensitivity of detecting changes in hepatocyte ATP levels because of the higher "background". This problem was overcome by first removing the erthrocytes by aspiration and "fixing" hepatocyte ATP with releasing reagent added directly to the attached cells.

Figure 4 shows the results using this procedure. The time required for 50% recovery in ATP levels in hepatocytes, treated with either 0.25 or 1.0 mM KCN for 10 min and then removed by replacement of the medium, to control cell ATP levels was estimated to be 9 min. LDH release, also measured in these experiments as additional evidence of reversibility, was 2.5% in flasks 4 hr after KCN had been removed by aspiration of the medium, compared with 11.6% in flasks in which KCN had not been removed. These observations confirmed that ATP was the indicator of choice among those evaluated.

Antidotal Action

The effects of classical antidotes on cyanide-induced toxicity to hepatocytes in the coincubation system were documented to validate the system for antidotal studies and to gain insight into the mechanisms of action of these antidotes at the cellular and molecular levels. The antidotes chosen for study were 4-dimethylaminophenol (DMAP), sodium nitrite ($NaNO_2$), cobaltous chloride ($CoCl_2$), sodium thiosulfate ($Na_2S_2O_3$), and a combination of $NaNO_2$ and $Na_2S_2O_3$. These antidotes are all effective in preventing lethality from massive cyanide poisoning, but by widely differing mechanisms (1-5).

All of the antidote treatments antagonized cyanide-induced depression of hepatocyte ATP in the coincubation system in a concentration-dependent manner (7,8). From the data in Table 1, the relative potency of these antidotes in the system is inferred to be, on a mM basis, DMAP > $CoCl_2$ >> $NaNO_2$ ≈ $Na_2S_2O_3$. The ranking is qualitatively similar to the relative potency of the antidotes *in vivo* for preventing lethality in mice, the only species for which published data on these four antidotes were available for comparison (27-29). These data indicate that the following ip doses (in mmol/kg) prevent lethality: $CoCl_2$, 0.08-0.25; DMAP, 0.29; $NaNO_2$, 1.0-1.4; and $Na_2S_2O_3$, 6.3-8.0. This compares favorably with the *in vitro* potency ranking (Table 2). The combination of $Na_2S_2O_3$ and $NaNO_2$ was more effective than either alone, as has been demonstrated *in vivo* (30).

The results of these mechanistic studies with $NaNO_2$ and DMAP indicate quite conclusively that the reversal of ATP depression by cyanide is dependent on the capability of the antidotes to form methemoglobin, based on the following: (a) The antidotes produced substantial methemoglobin in the system with KCN absent; (b) erythrocytes were required in the system

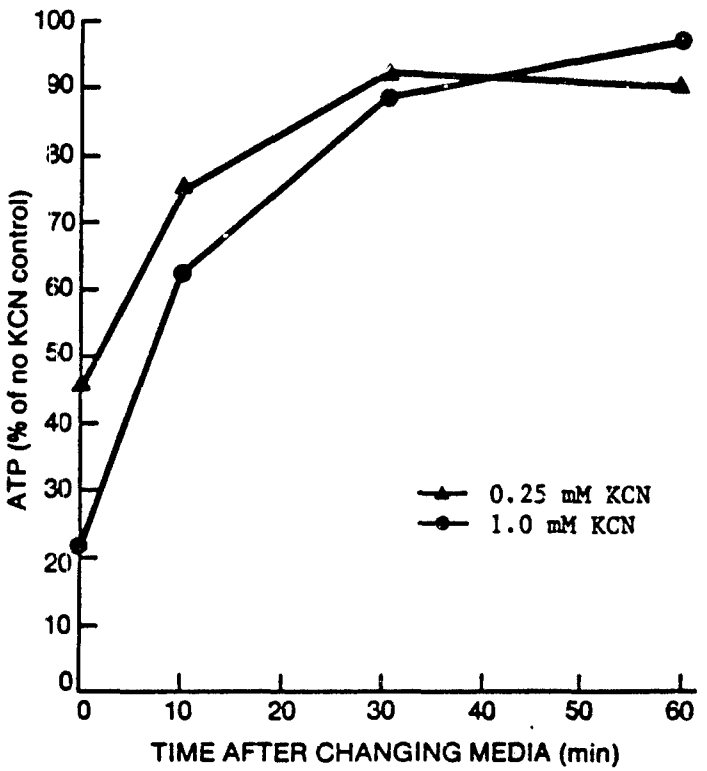


Figure 4. Reversal of ATP Depression on Replacement of KCN-Containing Medium After 10 Minutes with Fresh Medium Containing No KCN.

Table 1

REVERSAL OF ATP DEPRESSION IN HEPATOCYTES BY CYANIDE ANTIDOTES[a]

Antidote	Concn. (mM)	ATP Level (μM)[b] 0 mM KCN	ATP Level (μM)[b] 1.0 mM KCN	% of No-KCN Control[c]
None		11.7 ± 2.0[d]	3.0 ± 1.4	24.9 ± 11.9
DMAP	0.05	12.2 ± 2.2	4.2 ± 1.0	30.4 ± 21.4
	0.10	12.0 ± 2.9	7.1 ± 2.4	58.9 ± 10.2*[e]
	0.25	11.9 ± 2.2	8.2 ± 1.6	68.9 ± 9.9*
$CoCl_2$	0.10	12.2 ± 2.9	4.2 ± 3.1	32.3 ± 18.4
	0.25	11.6 ± 2.8	9.4 ± 2.0	84.3 ± 8.5*
$NaNO_2$	2.0	11.6 ± 1.7	3.6 ± 1.2	30.7 ± 6.2
	5.0	10.9 ± 2.3	5.8 ± 2.0	54.2 ± 21.8*
$Na_2S_2O_3$	4.0	11.6 ± 2.4	3.8 ± 0.4	33.5 ± 8.1
	10.0	11.2 ± 3.0	6.6 ± 2.3	62.3 ± 16.5*
$NaNO_2$ + $Na_2S_2O_3$	2.0 4.0	11.9 ± 2.0	6.7 ± 0.4	57.5 ± 9.5*
$NaNO_2$ + $Na_2S_2O_3$	5.0 4.0	11.4 ± 2.2	7.9 ± 1.2	70.4 ± 8.9*
$NaNO_2$ + $Na_2S_2O_3$	2.0 10.0	10.6 ± 2.9	7.8 ± 1.5	77.1 ± 20.0*
$NaNO_2$ + $Na_2S_2O_3$	5.0 10.0	10.6 ± 2.0	8.2 ± 0.8	78.6 ± 9.8*

[a] 1.0 mM KCN added to all flasks. Antidote added 10 min after KCN.

[b] Measured 60 min after addition of antidote.

[c] Respective 0 mM KCN flasks served as controls.

[d] Data are the means ± SD of three or four experiments.

[e] * = Significant increase ($p < 0.05$) relative to 1.0 mM KCN-treated flasks.

Table 2

COMPARISON OF ANTIDOTE EFFECTIVENESS
IN VITRO AND IN VIVO

Antidote	In Vitro[a] (mM)	In Vivo[b] (mmol/kg)
$CoCl_2$	0.10-0.25	0.08-0.25
DMAP	0.10	0.29
$NaNO_2$	2.0-5.0	1.0-1.4
$Na_2S_2O_3$	4.0-10.0	6.3-8.0

[a] Coincubation system using rat hepatocytes and erythrocytes. Concentration or range at which a statistically significant recovery in ATP levels occurred.

[b] Doses given ip to mice (27-29).

for reversal of cyanide-induced ATP depression to occur; (c) cyanmethemoglobin was produced at approximately stoichiometric levels with the quantity of KCN originally added; and (d) in the absence of $Na_2S_2O_3$, thiocyanate formation was insignificant (< 1.0%). These results parallel those of earlier investigators showing that these antidotes also reverse cytochrome oxidase inhibition by cyanide *in vitro* and *in vivo* (19-21,31). The results here do not exclude the possibility that other mechanisms may also be involved *in vivo*, as has been proposed for $NaNO_2$ (5). But these mechanisms most certainly do not involve formation of thiocyanate in the absence of added $Na_2S_2O_3$ (Table 3). Also, the ability to correlate methemoglobin levels with protection against lethality (in this case in mice and dogs) argues against this point of view and in support of the methemoglobinemia hypothesis (31,32).

Although this interpretation is consistent with the generally held mode of action of these antidotes from *in vivo* studies, we found one discrepancy: methylene blue did not prevent induction of methemoglobin in the presence of $NaNO_2$ as Holmes and Way (33) reported occurs in the mouse, which was the basis for the suggestion (5) that $NaNO_2$ was effective by some other mechanism. The differences may lie (a) in the use of different species, as the mouse erythrocyte is known to possess a much higher methemoglobin reductase activity, inducible with methylene blue, than the rat (34,35), or (b) in the experimental protocols or conditions *in vitro* and *in vivo*. (The *in vitro* experiment was limited to a 60-min incubation with excess $NaNO_2$, under which conditions little reductase activity is detected with methylene blue present, and the *in vivo* experiments cited employed a prophylactic rather than a therapeutic protocol as used *in vitro*.) However, it is important to recognize that the cycling of the hemoglobin-methemoglobin reaction by combination of $NaNO_2$ and methylene blue can be shifted to the right when cyanide is present because of the formation of a very stable methemoglobin-cyanide complex interrupting the cycle. Thus, to interpret the *in vivo* experiments as evidence against the methemoglobinemia hypothesis, one must be certain that not only is methemoglobin formation suppressed before the addition of cyanide, but also that cyanmethemoglobin is not present in stoichiometric quantities in animals that survived the cyanide treatment. It is not clear from the literature whether data on this point are available.

In the case of $CoCl_2$, omission of erythrocytes from the medium did not prevent antagonism by $CoCl_2$, and the antidote produced no cyanmethemoglobin in their presence (Table 3). Inhibition of methemoglobin reductase, considered *in vivo* as a possible mechanism of cobalt-mediated antagonism (36), is clearly not applicable *in vitro* and is unnecessary for effectiveness. No significant amount of SCN^- was formed with $CoCl_2$, indicating that cobalt(II)-enhanced sulfurtransferase activity (e.g., possibly through increases in glutathione levels) (37) is not a factor in antagonism. It seems highly probable, then, that effectiveness of the antidote in the coincubation system is due to direct complexation of Co ion with cyanide in the medium, as has been proposed to occur *in vivo* (27,38,39). Since Co(II) forms a hexacyano complex with CN^- (38), 0.25 mM $CoCl_2$ should reverse cyanide-induced ATP depression when the KCN concentration in the

Table 3

DISPOSITION OF CYANIDE IN THE COINCUBATION SYSTEM[a]

Antidote	Concn. (mM)	ATP (μM)[b]	% Cyanmethemoglobin[c]	SCN^- (μM)
None		2.8 ± 1.5	0.0	7.7 ± 3.2
DMAP	0.05	4.9 ± 1.1	64.3 ± 5.8	--[d]
	0.25	8.3 ± 2.0	83.3 ± 10.8	4.6 (5.0,4.1)
$NaNO_2$	2.0	3.6 (2.4,4.8)	38.5 (38.4,38.6)	--[d]
	5.0	5.2 ± 1.8	84.6 ± 7.4	7.6 (10.1,5.0)
$Na_2S_2O_3$	10.0	6.4 ± 2.6	3.0 ± 5.2	591 ± 184
$NaNO_2$ + $Na_2S_2O_3$	2.0 + 10.0	6.8 (6.6,6.9)	56.9 (81.9,31.8)	--[d]
$NaNO_2$ + $Na_2S_2O_3$	5.0 + 10.0	8.2 ± 2.5	84.1 ± 27.5	381 (402,358)
$CoCl_2$	0.25	9.5 ± 2.5	3.8 ± 6.6	10.2 (20.4,0.0)

[a] Antidotes were added 10 min after KCN, and the incubation continued for 60 min more, at which point ATP, cyanmethemoglobin, and SCN^- were quantitated. Data are either the means ± SD of four experiments or the means from two experiments with individual values in parentheses.

[b] Control ATP levels = 9.5 ± 3.4 μM for the four experiments involved. Not all antidotes were assayed in the same experiments.

[c] In cyanide-containing flasks. Initial hemoglobin content in the medium = 1.6 g/dL.

[d] Not determined.

system is 1.0 mM, whereas 0.10 mM $CoCl_2$ should not. This is indeed what was observed (Table 1). Analytical proof for the formation of the hexacyano complex and identification of the oxidation state of the cobalt ion were not sought in the work described here. Details of the mechanism of this reaction are also not present in the literature. Such experiments are needed for a definitive conclusion that the postulated mechanism is sufficient to explain cobalt's mode of action.

With thiosulfate, reversal of ATP depression in the coincubation system occurred concurrently with conversion of CN^- to SCN^-; cyanmethemoglobin was not appreciably formed (Table 3). Conversion of CN^- to SCN^- with this antidote was stoichiometric (not shown in table). Reversal of ATP by thiosulfate and SCN^- formation occurred with hepatocytes alone in the system and even when albumin was omitted from the medium. Presumably liver rhodanese, an enzyme with high substrate specificity and activity with thiosulfate, is responsible for the sulfurtransferase activity. Several investigators have cautioned against this interpretation outright, primarily based on experimental evidence showing that thiosulfate does not readily penetrate cell membranes and is not readily accessible to rhodanese located in the mitochondrial matrix (40-42). Furthermore, sulfane sulfur metabolism is complex and other enzymes may also be involved, making it exceedingly difficult to resolve the precise mechanism (1). Whatever the enzyme or enzymes involved and the mechanism of sulfur transfer, it is clear from the results obtained here that intact hepatocytes do have the capability for facilitating sulfane sulfur transfer from thiosulfate.

An unexpected and interesting outcome of these studies was that erythrocytes significantly increased the rate of antagonism of ATP depression by cyanide with thiosulfate antidote. Occasional reports of a rhodanese-like sulfurtransferase activity in erythrocytes have appeared in the literature (40,43,44), but the activity assayed as rhodanese was very small and uncharacteristically labile (45). Whatever the identity of the active component, erythrocytes in our system also exhibited sulfurtransferase activity with thiosulfate in the absence of hepatocytes, thereby confirming these earlier reports.

A few other experiments were conducted in an attempt to characterize erythrocyte sulfurtransferase. The enzyme, previously detected in rat and human erythrocytes by others and called rhodanese, has been noted to be uncharacteristically labile and may have some other differences. The erythrocyte enzyme here saturated at between 10 and 25 mM thiosulfate, well below that for the liver enzyme at physiologic pHs. Components in the Waymouth's medium, presumably S-containing amino acids or glutathione, facilitated sulfurtransferase with the intact cell, whereas little activity was obtained when a simple potassium phosphate buffer was employed at the same pH. Sodium sulfite inhibited the activity, suggesting that the active entity might actually be thiosulfate reductase, not rhodanese. When reduced glutathione, a substrate for thiosulfate reductase, was added to the medium, however, the expected enhancement was not observed, leaving the matter of the identity of the enzyme still in doubt. Lysing the cells

greatly increased the sulfur transfer rate, localizing the active site or component to the cytosol or inside surface of the plasma membrane. However, clarification is needed as to whether thiosulfate, in giving up its labile sulfur atom, actually penetrates the plasma membrane, and if it does, the pathways and enzymes involved.

It is not possible to comment definitively on the relative significance of the different sulfurtransferase activities measured here for in vivo metabolism even if a comprehensive study of activity vs. hepatocyte and erythrocyte content in the flask were carried out at 0.2 mM KCN, the dose corresponding to a lethal dose in rat blood (see above). Organ perfusion studies are better suited for this purpose. Some observations on the data here are instructive, however. The contents of hepatocytes (1.6×10^6 viable cells) and erythrocytes (0.4 ml of whole blood equivalents) used in the coincubation system display about equal sulfurtransferase activity with cyanide under the experimental conditions (6). Assuming 1.1×10^8 parenchymal cells/g wet weight of liver (46), an average liver weight of 14 g (47), and 18 ml of blood, one may estimate that the liver in an intact young-adult male rat would convert CN^- to SCN^- about 30 to 35 times faster than would erythrocytes with thiosulfate antidote, and that cyanide detoxication would occur preferentially in that organ and in kidney, which . . also highly active (1). However, liver sulfurtransferase activity is at least fivefold lower in dog and human hepatocytes than is noted in rodent hepatocytes. This is in contrast to erythrocytes from these species and from rats, which exhibited in our hands approximately twofold lower activity (8). In dogs, detoxication of CN^- to SCN^- in the bloodstream with thiosulfate infusion occurs within a few minutes (48,49). Sylvester et al. (49) also demonstrated that the apparent volume of distribution of the compartment in which cyanide is converted to SCN^- is approximately equal to the blood volume. From these observations they concluded that conversion of CN^- to SCN^- occurs primarily in blood, possibly through the intervention of serum albumin, as proposed by Vennesland et al. (50), or adjacent tissue. The results and analysis here suggest that erythrocytes could have a potentially significant role in CN^- detoxication, at least in some species, including human.

In contrast to the clear evidence for a sulfurtransferase activity in erythrocytes, we were unable to find conclusive evidence for significant activity with serum albumin alone. Albumin did give modest enhancement of ATP recovery and $CN^- \rightarrow SCN^-$ conversion in the coincubation system, but we found only minor enhancement when albumin content in the medium was increased from 0.2% to 5.0% with hepatocytes alone (~ 1.0 nmol/ml/min conversion rate with 1.0 mM KCN and 10 mM thiosulfate present) and none with erythrocytes or culture medium only in the system. We must conclude that participation by albumin in the overall transfer of sulfur from thiosulfate to cyanide is minor, if it occurs at all.

Interspecies Comparisons

The adoption of hepatocytes and erythrocytes for use in the coincubation system allows comparative studies to be performed, since these cell types are readily isolated from different mammalian species, including human. A limited number of experiments with cyanide and its antidotes was conducted with cells isolated from beagle, rhesus monkey, and human livers as tissues were made available from terminated studies conducted elsewhere at SRI or from kidney and heart/lung transplants at local hospitals with which SRI has arrangements for acquiring tissues donated for research.

The results of those experiments showed that the action of cyanide and its potency were very similar in hepatocytes from these three species compared with rat hepatocytes, but antidotal potency was not in all cases. Thus, ATP levels were depressed and LDH release was increased as a function of increasing KCN concentration in the medium, similarly to results with rat hepatocytes, with apparently minor differences for the most part. Insufficient replicates were performed for definitive conclusions on relative susceptibility of hepatocytes from the different species to cyanide action, but the differences that were recorded were not dramatic. Likewise, $NaNO_2$ (with or without $Na_2S_2O_3$) and/or DMAP and $CoCl_2$ at concentrations effective with rat hepatocytes in the coincubation system were similarly effective with hepatocytes from rhesus monkey and humans in which these antidotes were tested. In contrast, $Na_2S_2O_3$ was ineffective at the same concentration normally used in the rat hepatocyte-erythrocyte system. Human liver is known to have lower rhodanese enzyme activity than rat liver (51,52), and the results in vitro are consistent with this observation. One may conclude from this that thiosulfate alone as an antidote may not be as effective in humans as it is in rodents. Certainly, extrapolations of the response in rodents to the human situation must be viewed cautiously.

Other Experiments

The coincubation system was applied to the screening of newer antidotes for cyanide toxicity and to the detection of methemoglobin formation by metabolites of drugs for antileishmaniasis, a special application for which the system should be well suited. In the latter application, an involvement of metabolites in methemoglobinemia has been deduced from whole-animal studies, and adaptation of an in vitro approach for metabolite identification and elucidation of the mechanism in more detail would be beneficial.

Five new compounds were screened in the coincubation system for their capability in preventing depression of rat hepatocyte ATP by 1.0 mM KCN. These were the hydrates of cis-diaquo-bis(2,2'-bipyridyl)Co(III) triperchlorate (BK57874), 2-aminoethanethiol (BE 15684), 3-(4-aminobutylamino)propyl mercaptan (BK71025), 2-(3-aminopropylamino)ethyl mercaptan (BK71365), and 2-(5-aminopentylamino)ethyl mercaptan (BK 73869). The cobaltic compound was efective at 1.0 mM, the lowest concentration tested.

Two other antidotes, BK 73869 and BK 71365, effected nearly complete and 50% recovery of ATP to control flask levels, respectively, at 5.0 mM in the medium. The in vitro results with the cobaltic antidote are characteristic of this class of compounds and indicate promise as a cyanide antidote. A titration of cyanide present to antidote added was not conducted, but would be expected to be similar to results with $CoCl_2$. In addition, determination of the relative rates for reversal of ATP depression with each cobalt antidote and cytotoxic potenials are needed as part of the assessment as to which cobalt compound is better.

The most effective aminoalkylthiol that we evaluated, BK 73869, is the hydrolysis product of the corresponding phosphothioic acid. This compound is the most effective of the over 200 sulfur-containing radioprotective compounds tested. The results from in vivo tests in mice indicate that the 5-carbon separation of the amino groups is optimal for antagonism (53). The results here suggest further that the thiol itself may have some protective effect. The corresponding phosphorothioic acids need to be tested in the coincubation system for comparison to sort out the factors that contribute most to effectiveness and to gain insight into the mechanism.

Evaluation of Other Drugs for Methemoglobinemia

Two aminoquinoline drugs, WR 6026 (4-methyl-8-diethylaminohexylquinoline) and WR 2975 (primaquine), were also examined for their capability to induce methemoglobin formation in the coincubation system. The experiment was prompted out of a failure to observe a structure-activity correlation between responses in a human macrophage assay for antileishmaniasis and a hamster model (54). The discrepancy was tentatively attributed to differences in metabolism in vivo that were not reproduced in the human macrophage system. Because liver plays a role in the metabolism of these drugs, and metabolism is implicated in methemoglobin formation by them (a side effect that occurs in parallel with antileishmaniasis activity but at higher doses), this hypothesis could be tested in the coincubation system.

Under the normal incubation conditions that we use, WR 6026, which is the more active in vivo, induced appreciable methemoglobin formation at 1.0 and 10.0 mM, whereas WR 2975 did at 10.0 mM but not at 1.0 mM. The medium was turbid in those dishes in which methemoglobin was induced by either drug, apparently from lysing of erythrocytes, as the hepatocyte cultures themselves were seen to be unchanged by light microscopy. The same degree of activity was obtained at 0.10 mM, however, if hepatocytes were omitted from the system.

In an attempt to increase sensitivity without attendant lysing, the experiment was repeated at drug concentrations of 0.10 mM (and lower) and at one-fifth the normal RBC content in the medium. Methemoglobin levels of ~10% were induced at 30 min with WR 6026 only under these conditions, but the levels were not increased substantially on longer incubations (up to 120 min). The results are reminiscent of those obtained with methylene

blue presented earlier, which antidote, though a methemoglobin inducer, converts a maximum of 20% of hemoglobin only. Possibly, as with methylene blue, methemoglobin reductase is also activated under these conditions, limiting the level of methemoglobin that can be formed.

Conclusions

It is concluded from the above that the principal research objectives set forth at the beginning of the contract have been essentially accomplished. Specifically, a novel coincubation system, comprising hepatocytes in monolayer culture and erythrocytes suspended in the incubation medium, has been developed and successfully validated for the study of cyanide and antidotal mechanisms. Isolated hepatocytes have been shown to respond to cyanide exposure in the system in a manner consistent with expectations from *in vivo* studies, both qualitatively and quantitatively. The data generated support for the hypothesis that inhibition of cytochrome oxidase is responsible for the toxic sequelae observed. The concentration range in which inhibition of mitochondrial O_2 consumption occurs overlaps the range in blood plasma *in vivo* in rats at which lethality is observed, as should be the case for a general cytotoxin like cyanide. Classical antidotes produce an antagonistic effect on cyanide action in the coincubation system, with similar potency and with the same modes of action expected of them, based on the literature in general.

From these observations it is further concluded that the coincubation system described herein is valid as a preliminary screen for cyanide antidotes from drug classes shown to be effective in the system and for studies on antidotal action at the molecular and cellular levels. Interspecies comparison of antidotal effectiveness is a particularly valuable application. Likewise, the development of efficacy/toxicity ratios, although not conducted in the present studies, is practical and warranted for further guidance on antidote selection for comprehensive testing. The extension of the coincubation system approach to other target cell types (brain and heart) would enhance its versatility and value considerably and is encouraged.

LITERATURE CITED

1. Westley, J., H. Adler, L. Westley, and C. Nishida. The sulfurtransferases. Fundam. Appl. Toxicol. 3, 377-382 (1983).

2. Way, J. L. Cyanide antagonism. Fundam. Appl. Toxicol. 3, 383-386 (1983).

3. Weger, N. P. Treatment of cyanide poisoning with 4-dimethylaminophenol (DMAP) - Experimental and clinical overview. Fundam. Appl. Toxicol. 3, 387-396 (1983).

4. Ballantyne, B. Artifacts in the definition of toxicity by cyanides and cyanogens. Fundam. Appl. Toxicol. 3, 400-408 (1983).

5. Way, J. L., D. Sylvester, R. L. Morgan, G. E. Isom, G. E. Burrows, C. B. Tamulinas, and J. L. Way. Recent perspectives on the toxicodynamic basis of cyanide antagonism. Fundam. Appl. Toxicol. 4, S231-S239 (1984).

6. Tyson, C. A. Mechanism of Chemical Action and Treatment of Cyanide Poisoning. Annual Report #1, Contract DAMD17-82-C-2211, SRI International, Menlo Park, CA, October 1983.

7. Tyson, C. A. Mechanism of Chemical Action and Treatment of Cyanide Poisoning. Annual Report #2, Contract DAMD17-82-C-2211, SRI International, Menlo Park, CA, October 1984.

8. Tyson, C. A. Mechanism of Chemical Action and Treatment of Cyanide Poisoning. Annual Report #3, Contract DAMD17-82-C-2211, SRI International, Menlo Park, CA, June 1986.

9. Kariman, K., and D. S. Burkhart. Reaction of cyanide with cytochrome aa_3 in isolated perfused rat head *in situ*. Biochem. J. 233, 187-191 (1986).

10. Egekeze, J. O., and F. W. Oehme. Blood and liver cyanide concentrations in rats poisoned with oral doses of potassium cyanide. Toxicol. Lett. 3, 243-247 (1979).

11. Yamamoto, K., Y. Yamamoto, H. Hattori, and T. Samori. Effects of routes of administration on the cyanide concentration distribution in the various organs of cyanide-intoxicated rats. Tokoku J. Exp. Med. 137, 73-78 (1982).

12. Schubert, J., and W. A. Brill. Antagonism of experimental cyanide toxicity in relation to the *in vivo* activity of cytochrome oxidase. J. Pharmacol. Exp. Ther. 162, 352-359 (1968).

13. Isom, G. E., and J. L. Way, Lethality of cyanide in the absence of inhibition of liver cytochrome oxidase. Biochem. Pharmacol. 25, 605-608 (1976).

14. Isom, G. E., G. E. Burrows, and J. L. Way, Effect of oxygen on the antagonism of cyanide intoxication-cytochrome oxidase, in vivo. Toxicol. Appl. Pharmacol. 65, 250-256 (1982).

15. Solomonson, L. P. Cyanide as a metabolic inhibitor. In Cyanide in Biology. B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley, and F. Wissing (eds.), Academic Press, New York, 1981, pp. 11-28.

16. Story, D. L., S. J. Gee, C. A. Tyson, and D. H. Gould. Response of isolated hepatocytes to organic and inorganic cytotoxins. J. Toxicol. Environ. Health 11, 483-501 (1983).

17. Kachmar, J. F., and D. W. Moss. Enzymes. In Fundamentals of Clinical Chemistry. N. W. Tietz (ed.), W. B. Saunders Co., Philadephia, 1976, pp. 565-698.

18. Ozawa, K. Energy metabolism. In Pathophysiology of Shock, Anoxia, and Ischemia. R. A. Cowley and B. F. Trump (eds.), Williams and Wilkins, Baltimore, 1982, pp. 74-73.

19. Albaum, H. G., J. Tepperman, and O. Bodansky. A spectrophotometric study of the competition of methemoglobin and cytochrome oxidase for cyanide in vitro. J. Biol. Chem. 163, 641-647 (1946).

20. Estler, C.-J. Stoffwechselveränderugen des gehirns im verlauf der nicht letalen kaliumcyanidvergiftung und ihre beeinflussung durch cyanideantagoniston. Arch. Exp. Pathol. Pharmakol. 251, 413-423 (1965).

21. Schubert, J., and W. A. Brill. Antagonism of experimental cyanide toxicity in relation to the in vivo activity of cytochrome oxidase. J. Pharmacol. Exp. Ther. 162, 352-359 (1968).

22. Piantadosi, C. A., and A. L. Sylvia. Cerebral cytochrome a,a_3 inhibition by cyanide in bloodless rats. Toxicology 33, 67-79 (1984).

23. Pettersen, J. C., and S. D. Cohen. Antagonism of cyanide poisoning by chlorpromazine and sodium thiosulfate. Toxicol. Appl. Pharmacol. 81, 265-273 (1985).

24. Siesjo, B. K. Brain Energy Metabolism. John Wiley & Sons, New York, 1978, pp. 179-186.

25. Mela, L. Mitochondrial function in shock, ischemia, and hypoxia. In Pathophysiology of Shock, Anoxia, and Ischemia. R. A. Cowley and B. F. Trump (eds.), Williams and Wilkins, Baltimore, 1982, 84-95.

26. Hansen, A. J. Effect of anoxia on ion distribution in the brain. Physiol. Rev. 65, 101-148 (1985).

27. Frankenberg, L., and B. Sorbo. Effect of cyanide antidotes on the metabolic conversion of cyanide to thiocyanate. Arch. Toxicol. 33, 81-89 (1975).

28. Isom, G., and J. L. Way. Cyanide intoxication, protection with cobaltous chloride. Toxicol. Appl. Pharmacol. 24, 449-456 (1973).

29. Kruszyna, R., H. Kruszyna, and R. P. Smith. Comparison of hydroxylamine, 4-dimethylaminophenol and nitrite protection against cyanide poisoning in mice. Arch. Toxicol. 49, 191-202 (1982).

30. Chen, K. K., and C. L. Rose. Nitrite and thiosulfate therapy in cyanide poisoning. J. Am. Med. Assoc. 149, 113-119 (1952).

31. Klimmek, R., H. Fladerer, and N. Weger. Circulation, respiration and blood homeostasis in cyanide-poisoned dogs after treatment with 4-dimethylaminophenol or cobalt compounds. Arch. Toxicol. 43, 121-131 (1979).

32. Smith, R. P., and R. E. Gosselin. On the mechanism of sulfide inactivation by methemoglobin. Toxicol. Appl. Pharmacol., 8, 159-172 (1966).

33. Holmes, R. K., and J. L. Way. Mechanism of cyanide antagonism by sodium nitrite. The Toxicologist 2, 182 (1982).

34. Stolk, J. M., and R. P. Smith. Species differences in methemoglobin reductase activity. Biochem. Pharmacol. 15, 343-351 (1966).

35. Smith, R. P., A. A. Alkaitis, and P. R. Shafer. Chemically induced methemoglobinemias in the mouse. Biochem. Pharmacol. 16, 317-328 (1967).

36. Hagler, L., and R. I. Coppes, Jr. Inhibition of methemoglobin and metmyoglobin reduction by cobalt. Biochem. Pharmacol. 31, 1779-1782 (1982).

37. Ivankovich, A. D., B. Braverman, R. P. Kanuru, H. J. Heyman, and R. Paulissian. Cyanide antidotes and methods of their administration in dogs: a comparative study. Anesthesia 52, 210-216 (1980).

38. Evans, C. L. Cobalt compounds as antidotes for hydrocyanic acid. Br. J. Pharmacol. 23, 455-475 (1964).

39. Smith, R. P. Cobalt salts: Effects in cyanide and sulfide poisoning and on methemoglobinemia. Toxicol. Appl. Pharmacol. 15, 505-516 (1969).

40. Himwich, W. A., and J. P. Saunders. Enzymatic conversion of cyanide to thiocyanate. Am. J. Physiol. 153, 348-354 (1948).

41. Greville, G. D., and J. B. Chappell. The latent rhodanese of isolated rat-liver mitochondria. Biochim. Biophys. Acta 33, 267-269 (1959).

42. Rutkowski, J. V., B. D. Roebuck, and R. P. Smith. Effects of protein-free diet and food deprivation on hepatic rhodanese activity, serum proteins and acute cyanide lethality in mice. J. Nutr. 115, 132-137 (1985).

43. Toida, T. Blood cyanide and thiocyanate. Teikyo Igaku Zasshi 6, 163-169 (1983).

44. Coltorti, M., and G. Giusti. Caratteristiche biochimiche della rodanese delle emazie umane. Boll. Soc. Ital. Biol. Sper. 32, 256-261 (1956).

45. Westley, J. Rhodanese. Adv. Enzymol. 39, 327-368 (1973).

46. Seglen, P. O. Preparation of isolated rat liver cells. III. Enzymatic requirements for tissue dispersion. Exp. Cell Res. 82, 391-398 (1973).

47. Dilley, J. V., C. A. Tyson, R. J. Spanggord, D. P. Sasmore, G. W. Newell, and J. C. Dacre. Short-term oral toxicity of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine (1:0.62) mixture in mice, rats, and dogs. J. Toxicol. Environ. Health 9, 587-610 (1982).

48. Christel, D., P. Eyer, M. Hegemann, M. Kiese, W. Lörcher, and N. Weger. Pharmacokinetics of cyanide in poisoning of dogs, and the effect of 4-dimethylaminophenol or thiosulfate. Arch. Toxicol. 38, 177-189 (1977).

49. Sylvester, D. M., W. L. Hayton, R. L. Morgan, and J. L. Way. Effects of thiosulfate on cyanide pharmacokinetics in dogs. Toxicol. Appl. Pharmacol. 69, 265-271 (1983).

50. Vennesland, B., P. A. Castric, E. E. Conn, L. P. Solomonson, M. Volini, and J. Westley. Cyanide metabolism. Fed. Proc. 41, 2639-2648 (1982).

51. Lang, K. Die rhodanbildung intierkorper. Biochem Z. 259, 243-256 (1933).

52. Reinwein, D. Die verteilung der thiosulfat-schwefeltransferase und des rhodanids im menschlichen und tierischen organismus. Hoppe-Seyler's Z. Physiol. Chem. 326, 94-101 (1961).

53. Davidson, D. E., D. Davis, and C. J. Canfield, Chemoprophylaxis of cyanide intoxication. Fourth Annu. Chem. Def. Bioscience Rev., USAMRDC, Aberdeen Proving Ground, MD, 30 May to 1 June (198[illegible].

54. Berman, J. D., and L. S. Lee. Activity of 8-aminoquinolines against Leishmania tropica with human macrophages in vitro. Am. J. Trop. Med. Hyg. 32, 753-759 (1983).

BIBLIOGRAPHY OF PUBLICATIONS UNDER THE CONTRACT

Gee, S. J., C. E. Green, and C. A. Tyson. Species differences in the metabolism of amphetamine by isolated hepatocytes. The Toxicologist 4, 127 (1984).

Tyson, C. A., S. J. Gee, and S. E. Le Valley. A new mechanism for sulfane S transfer from sodium thiosulfate to cyanide. The Toxicologist 5, 79 (1985).

Tyson, C. A., S. J. Gee, S. E. Le Valley, and C. E. Green. Investigations of cyanide antidote action at the cellular level. Proc. Fifth Annual Chemical Defense Bioscience Review, Columbia, MD, May 29-31 (1985).

Gee, S. J., S. E. LeValley, and C. A. Tyson. Application of a hepatocyte-erythrocyte coincubation system to studies of cyanide antidotal mechanisms. Toxicol. Appl. Pharmacol. 88, 24-34, 1987.

Gee, S. J., C. E. Green, and C. A. Tyson. Mechanism of cyanide-induced cytotoxicity in isolated hepatocytes. Fundam. Appl. Toxicol. (submitted for publication).

Tyson, C. A., S. J. Gee, and C. E. Green. Mechanism of cyanide-induced cytotoxicity. The Toxicologist 7, 64, 1987.

Tyson, C. A. Correspondence of results from hepatocyte studies with *in vivo* response. Toxicol. Ind. Health (in press).

LIST OF PERSONNEL THAT RECEIVED CONTRACT SUPPORT

Charles A. Tyson, Ph.D., Principal Investigator

Carol E. Green, Ph.D., Toxicologist

Shirley J. Gee, M.S., Toxicologist

Susanna E. Le Valley, B.S., Biochemist

Jack E. Dabbs, B.S., Cell Biologist

DISTRIBUTION LIST

Copies	
1	Commander U.S. Army Medical Research & Development Command ATTN: SGRD-RMI-S Fort Detrick Frederick, MD 21701
5	Commander U.S. Army Medical Research & Development Command ATTN: SGRD-PLE Fort Detrick Frederick, MD 21701-5012
12	Administrator Defense Technical Information Center ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1	Commandant Academy of Health Sciences, U.S. Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100
1	Dean, School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799